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<p>(54) Title: CHIMERIC NEOMORPHOGENESIS OF ORGANS BY CONTROLLED CELLULAR IMPLANTATION USING ARTIFICIAL MATRICES</p>		
<p>(57) Abstract</p> <p>A method and means for providing functional equivalents to organs wherein cells are grown on polymer scaffolding using cell culture techniques followed by transfer of the polymer-cell scaffold into a patient at a site appropriate for attachment, growth and function, after growth and vascularization. Once the structure is implanted and vascularization takes place, the resulting organ is a blend of the parenchymal elements of the donated tissue and vascular and matrix elements of the host. A key element of the method is the design and construction of the polymer scaffold using a material and shape that provides for attachment and growth of the cells such that an adequate exchange of nutrients, wastes and gases occurs by diffusion even within the inner layers of the cells, until such time as implantation and vascularization occur.</p>		

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1 transplantation of islet cells through injection of
2 isolated clusters of islet cells into the portal
3 circulation, with implantation in the vascular bed of
4 the liver. More recent experimental methods have
5 included encapsulation of pancreatic beta cells to
6 prevent immune attack by the host and injection of
7 fetal beta cells beneath the capsule of the kidney.
8 Although there is evidence of short term function,
9 long term results have been less satisfactory (D.E.R.
10 Sutherland, Diabetologia 20, 161-185 (1981); D.E.R.
11 Sutherland, Diabetologia 20, 435-500 (1981)). Currently
12 whole organ pancreatic transplantation is the
13 preferred treatment.

14 There are also many diseases which cause
15 significant scarring of the liver, ultimately causing
16 hepatic failure. There are no artificial support
17 systems for liver failure, so that, in the absence of
18 a successful transplant, liver failure always results
19 in the death of the patient. It has been estimated
20 that 30,000 people die of hepatic failure every year
21 in the United States, at a cost to society of \$14
22 billion dollars annually.

23 There are many diseases which are termed "inborn
24 errors of metabolism", including genetic defects that
25 result in defects of protein metabolism, defects of
26 amino acid metabolism, defects of carbohydrate
27 metabolism, defects of pyrimidine and purine
28 metabolism, defects of lipid metabolism, and defects
29 of mineral metabolism. A large number of these
30 diseases are based in defects within the liver itself.
31 Many of these patients have a structurally normal
32 liver or reasonably normal liver at the time diagnosis
33 is made. Many of the diseases, in fact, do not damage

1 large. For example, in the Baltimore area during 1973
2 the age adjusted incidence rates for all alcoholic
3 liver diseases per 100,000 population over 20 years
4 were: 36.3 for white males, 19.8 for white females,
5 60.0 for nonwhite males, and 25.4 for nonwhite
6 females. The morbidity for liver cirrhosis has been
7 reported to be twenty-eight times higher among serious
8 problem drinkers than amongst nondrinkers in a survey
9 of factory workers. There is a direct correlation
10 between the amount of alcohol consumed and the
11 incidence of cirrhosis. The mortality rates for
12 cirrhosis vary greatly from country to country,
13 ranging from 7.5 per 100,000 in Finland to 57.2 per
14 100,000 in France. In the U.S., the trend has been
15 alarming in terms of increasing incidence of alcoholic
16 cirrhosis and death. Between 1950 to 1974, deaths from
17 cirrhosis in the U.S. increased by 71.7% while deaths
18 from cardiovascular diseases decreased by 2%. At this
19 time, these patients have no options.

20 There are many other vital organ systems for
21 which there is no adequate means for replacement or
22 restoration of lost function. For example, in the
23 past, loss of the majority of intestine was a fatal
24 condition. Although patients can now be supported
25 totally with nutrition supplied via the veins, this is
26 thought of as a "half-way technology" because of the
27 many complications associated with this technique.
28 One problem is that, over time, many patients on total
29 parenteral nutrition develop irreversible liver
30 disease and die of their liver disease. Other
31 patients develop severe blood stream infections
32 requiring multiple removal and replacement procedures.
33 They may eventually lose all available veins and
34 succumb of malnutrition or die of infection.

1 Selective cell transplantation of only those
2 parenchymal elements necessary to replace lost
3 function has been proposed as an alternative to whole
4 or partial organ transplantation (P.S.Russell, Ann.
5 Surg. 201(3),255-262 (1985)). This has several
6 attractive features, including avoiding major surgery
7 with its attendant blood loss, anesthetic
8 difficulties, and complications. It replaces only
9 those cells which supply the needed function and,
10 therefore, problems with passenger leukocytes, antigen
11 presenting cells, and other cell types which may
12 promote the rejection process are avoided. Adding the
13 techniques of cell culture provides another set of
14 tools to aid in the transplantation process. The
15 ability to expand cell numbers with proliferation of
16 cells in culture, in theory, allows
17 autotransplantation of one's own tissue. For example,
18 hepatocyte injections into the portal circulation have
19 been attempted to support hepatic function. A recent
20 novel approach in which hepatocytes were attached to
21 collagen coated microcarrier beads prior to injection
22 into the peritoneal cavity demonstrated successful
23 implantation, viability of the implanted hepatocytes,
24 and function, as described by A.A.Demetriou, et al.,
25 Science 233,1190-1192 (1986).

26 Loss of other types of organ or tissue function
27 such as muscle or nervous tissue can also lead to
28 deforming illnesses and social tragedies. Methods of
29 muscle and nerve transfer have been developed by
30 surgeons through the last fifty years which are
31 ingenious in design. An example of a technique for
32 restoring nerve function has been to string dead nerve
33 fibers from nerve centers to places with lost nerve
34 function. Many other disorders of the nervous system

1 skin-equivalent. U.S. Patent No. 4,060,081, to Yannas
2 et al. discloses a multilayer membrane useful as
3 synthetic skin which is formed from an insoluble non-
4 immunogenic material which is nondegradable in the
5 presence of body fluids and enzymes, such as cross-
6 linked composites of collagen and a
7 mucopolysaccharide, overlaid with a non-toxic material
8 such as a synthetic polymer for controlling the
9 moisture flux of the overall membrane. U.S. Patent
10 No. 4,458,678 to Yannas et al. discloses a process for
11 making a skin-equivalent material wherein a fibrous
12 lattice formed from collagen cross-linked with
13 glycosaminoglycan is seeded with epidermal cells.

14 A disadvantage to the first two methods is that
15 the matrix is formed of a "permanent" synthetic
16 polymer. The '678 patent has a feature that neither
17 of the two prior patents has, a biodegradable matrix
18 which can be formed of any shape, using the
19 appropriate cells to produce an organ such as the
20 skin. Unfortunately, there is a lack of control over
21 the composition and configuration of the latter
22 matrices since they are primarily based on collagen.
23 Further, since collagen is degraded by enzymatic
24 action as well as over time by hydrolysis, the
25 degradation is quite variable. Moreover, the matrix
26 is completely infiltrated with cells and functional in
27 the absence of the moisture controlling polymer
28 overlay only when it is grafted onto the patient and
29 capillaries have formed a vascular network through the
30 entire thickness of the matrix. The limitation of
31 these matrices as a function of diffusion is discussed
32 in the article by Yannas and Burke in
33 J.Biomed.Mater.Res., 14, 65-81 (1980) at page 73.
34 Although the authors recognized that the pore size and

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1 blood vessels, nerves, and muscles, which functionally
2 resemble the naturally occurring organ.

3 It is a further object of the present invention
4 to provide a method and means for designing,
5 constructing and utilizing artificial matrices as
6 temporary scaffolding for cellular growth and
7 implantation.

8 It is a still further object of the invention to
9 provide biodegradable, non-toxic matrices which can be
10 utilized for cell growth, both in vitro and in vivo,
11 as support structures in transplant organs immediately
12 following implantation.

13 It is another object of the present invention to
14 provide a method for configuring and constructing
15 biodegradable artificial matrices such that they not
16 only provide a support for cell growth but allow and
17 enhance vascularization and differentiation of the
18 growing cell mass following implantation.

19 It is yet another object of the invention to
20 provide matrices in different configurations so that
21 cell behavior and interaction with other cells, cell
22 substrates, and molecular signals can be studied in
23 vitro.

24 Summary of the Invention

25 The present invention is a method and means
26 whereby cells having a desired function are grown on
27 polymer scaffolding using cell culture techniques,
28 followed by transfer of the polymer-cell scaffold into
29 a patient at a site appropriate for attachment, growth
30 and function, after attachment and equilibration, to
31 produce a functional organ equivalent. Success
32 depends on the ability of the implanted cells to

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1 and gases, so as to produce uniform cell growth and
2 proliferation. Theoretical calculations of the
3 maximum cell attachment suggest that fibers 30 microns
4 in diameter and one centimeter in length can support
5 125,000,000 cells and still provide access of
6 nutrients to all of the cells. Another advantage of
7 the biodegradable material is that compounds may be
8 incorporated into the matrix for slow release during
9 degradation of the matrix. For example, nutrients,
10 growth factors, inducers of differentiation or de-
11 differentiation, products of secretion,
12 immunomodulators, inhibitors of inflammation,
13 regression factors, biologically active compounds
14 which enhance or allow ingrowth of the lymphatic
15 network or nerve fibers, and drugs can be incorporated
16 into the matrix or provided in conjunction with the
17 matrix, in solution or incorporated into a second
18 biodegradable polymer matrix.

19 Cells of one or more types can be selected and
20 grown on the matrix. The matrix structure and the
21 length of time and conditions under which the cells
22 are cultured in vitro are determined on an individual
23 basis for each type of cell by measuring cell
24 attachment (only viable cells remain attached to the
25 polymers), extent of proliferation, and percent
26 successful engraftment. Examples of cells which are
27 suitable for implantation include hepatocytes and bile
28 duct cells, islet cells of the pancreas, parathyroid
29 cells, thyroid cells, cells of the adrenal-
30 hypothalamic-pituitary axis including hormone-producing
31 gonadal cells, epithelial cells, nerve cells, heart
32 muscle cells, blood vessel cells, lymphatic vessel
33 cells, kidney cells, and intestinal cells, cells

Brief Description of the Drawings

1

2 Figure 1 is a schematic of the process of the
3 present invention to produce a chimeric organ, in this
4 diagram, a liver, pancreas or intestine: (1) the
5 appropriate parenchymal cells are harvested,
6 dispersed, and seeded onto the polymer matrix in cell
7 culture, where attachment and growth occur and (2) a
8 partial hepatectomy is performed to stimulate growth
9 of the transplant and the polymer-cell scaffold is
10 then implanted into the recipient animal where
11 neovascularization, cell growth, and reabsorption of
12 the polymer matrix occurs.

13 Figure 2 are the chemical structures of polymers
14 which have been used for biodegradable cellular
15 matrices: (a) polygalactin; (b) polyorthoester; and
16 (c) polyanhydride.

17 Figure 3 is a diagram demonstrating the slow
18 release of biologically active factors from the
19 polymer matrix.

20 Figure 4 is a diagram of a technique to study in
21 vitro morphogenesis using biodegradable polymers,
22 cells, and matrix.

23 Figure 5 is a photograph (172x) of hepatocytes
24 attached to fibers of polyglactin 910 after 4 days in
25 culture. Cells are stained with Hematoxylin and
26 Eosin.

27 Figure 6 is a photograph of bile duct epithelial
28 cells cultured on polymer fibers for one month.

29 Figure 7 is a photograph (172X) of an implant of
30 hepatocytes from an adult rat donor into omentum. The
31 polymer-cell implant has been in place for 7 days
32 before sacrifice. Hepatocytes are healthy and several
33 mitotic figures can be seen. Blood vessels are

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1 Figure 14 is a photograph of bovine aortic
2 endothelial cells attached to polymer fibers after one
3 month in culture.

4 Figure 15 is a phase contrast photomicrograph
5 showing polymer fibers coated with mouse fetal
6 fibroblasts. The fibroblasts can be seen streaming
7 off the polymer fibers in a straight line onto the
8 culture dish.

9 Figure 16 is a phase contrast photomicrograph of
10 polymer fibers coated with mouse fetal fibroblasts.
11 These fetal fibroblasts have migrated off of the
12 polymer through media and have attached at the bottom
13 of the tissue culture plate.

14 Figure 17 is a scanning electron micrograph
15 (472x) of a polyanhydride fiber immersed in a
16 phosphate buffer solution, indicating that immersion
17 of polymer fibers in differing buffers can alter the
18 polymer surface and, therefore, influence cell
19 attachment and differentiation.

20 Figure 18 is a scanning electron micrograph
21 (493x) of polymer fibers coated with 1% gelatin,
22 showing that the polymer fibers can be coated with
23 cell adhesion agents to increase cell attachment.

24 Figure 19 is a perspective drawing of a
25 bioabsorbable polymer fiber used for growth of nerve
26 cells.

27 Figure 20a is a plan drawing of polymer spicules
28 seeded with heart muscle cells and implanted on the
29 myocardium of the heart.

30 Figure 20b is an enlarged plan view of a
31 spicule, as shown in Figure 20a.

32 Figure 21a is a cross sectional view of wells
33 containing various thicknesses of collagen (0, 3.0 mm,

1 are placed and the degree of alteration which they
2 have undergone.

3 3. Tissue cannot be implanted in volumes
4 greater than approximately one to three mm³, because
5 nutrition is supplied by diffusion until new blood
6 vessels form, and this distance is the maximum
7 distance over which diffusion can transpire until
8 angiogenesis occurs.

9 4. Cell shape is determined by cytoskeletal
10 components and attachment to matrix plays an important
11 role in cell division and differentiated function. If
12 dissociated cells are placed into mature tissue as a
13 suspension without cell attachment, they may have a
14 difficult time finding attachment sites, achieving
15 polarity, and functioning because they begin without
16 intrinsic organization. This limits the total number
17 of implanted cells which can remain viable to
18 organize, proliferate, and function.

19 The latter principle is a key point in the
20 configuration of the support matrices. For an organ
21 to be constructed in tissue culture and subsequently
22 successfully implanted, the matrices must have
23 sufficient surface area and exposure to nutrients such
24 that cellular growth and differentiation can occur
25 prior to the ingrowth of blood vessels following
26 implantation. After implantation, the configuration
27 must allow for diffusion of nutrients and waste
28 products and for continued blood vessel ingrowth as
29 cell proliferation occurs.

30 This method for replacing or supplementing lost
31 organ function has a number of advantages over either
32 pharmacologic manipulation or transplantation of whole
33 organs or parts of organs. Although great strides
34 have been made in these areas, the results of these

1 remaining 10% can be harvested and cultured. The
2 cells expand in a logarithmic fashion in culture. The
3 cells are cultured until suitable numbers of cells are
4 achieved, the cells are grown onto the appropriate
5 polymer scaffold, and placed back into the patient, to
6 be allowed to vascularize, grow and function as a
7 neointestine.

8 In the case of liver function replacement, it
9 may be possible to construct a cell-matrix structure
10 without the absolute need for hepatocyte proliferation
11 in culture. This hypothesis is based on the
12 observation that a high yield of hepatocytes can be
13 obtained from a small piece of liver. For example, in
14 experiments on 250 gm rats, it is known that the liver
15 weighs approximately 12 gm. At a 90% viability rate
16 this yields 2.5×10^8 viable hepatocytes. It is also
17 thought that only 10% of hepatic cell mass is
18 necessary for cell function. Therefore, for a 250 gm
19 rat, 1.2 gm of tissue is needed, an implant of
20 approximately 2.5×10^7 cells. This assumes no
21 proliferation in vivo. Implants into children as well
22 as adults are theoretically possible. An 8 month
23 child has a normal liver that weighs approximately 250
24 gm. That child would, therefore, need 25 gm of tissue
25 from a biopsy from a parent. An adult liver weighs
26 approximately 1500 gm, therefore, the biopsy would
27 only be about 1.5% of his liver or 5.0×10^8 cells.
28 Again, this assumes no proliferation. An adult would
29 need a larger biopsy which would yield about 2.5×10^8
30 cells. If these cells are attached with high
31 efficiency and implanted, proliferation in the new
32 host should occur. The resulting hepatic cell mass
33 should be adequate to replace needed function.

1 In distinct contrast to the prior art, the
2 present method uses a temporary scaffolding for
3 controlled growth and proliferation of cells in vitro,
4 followed by implantation of functional cells into
5 patients. The result is an organ which is
6 vascularized in vivo to allow growth of the cells in a
7 three-dimensional configuration similar to that of the
8 organ whose function they are replacing. Both the
9 design and construction of the scaffolding, as well as
10 the conditions of the initial cell culture, are used
11 to encourage cells to achieve their biological
12 potential and replicate the ontogeny of organ
13 formation which occurs in embryonic and fetal life.
14 As described herein, this technique is termed chimeric
15 neomorphogenesis.

16 The design and construction of the scaffolding
17 is of primary importance. The matrix should be shaped
18 to maximize surface area to allow adequate diffusion
19 of nutrients and growth factors to the cells. The
20 maximum distance over which adequate diffusion through
21 densely packed cells can occur appears to be in the
22 range of approximately 100 to 300 microns under
23 conditions similar to those which occur in the body,
24 wherein nutrients and oxygen diffuse from blood
25 vessels into the surrounding tissue. The actual
26 distance for each cell type and polymer structure must
27 be determined empirically, measuring cell viability
28 and function in vitro and in vivo. This determination
29 for bovine capillary endothelial cells in combination
30 with a collagen matrix will be described in detail in
31 a subsequent example.

32 The cells are initially cultured using
33 techniques known to those skilled in the art of tissue
34 culture. Once the cells have begun to grow and cover

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1 normally be achieved where nutrients are supplied
2 solely by diffusion.

3 All polymers for use in the present invention
4 must meet the mechanical and biochemical parameters
5 necessary to provide adequate support for the cells
6 with subsequent growth and proliferation. The
7 polymers can be characterized with respect to
8 mechanical properties such as tensile strength using
9 an Instron tester, for polymer molecular weight by gel
10 permeation chromatography (GPC), glass transition
11 temperature by differential scanning calorimetry (DSC)
12 and bond structure by infrared (IR) spectroscopy; with
13 respect to toxicology by initial screening tests
14 involving Ames assays and in vitro teratogenicity
15 assays, and implantation studies in animals for
16 immunogenicity, inflammation, release and degradation
17 studies.

18 In vitro cell attachment and viability can be
19 assessed using scanning electron microscopy,
20 histology, and quantitative assessment with
21 radioisotopes.

22 The configuration of the polymer scaffold must
23 have enough surface area for the cells to be nourished
24 by diffusion until new blood vessels interdigitate
25 with the implanted parenchymal elements to continue to
26 support their growth, organization, and function.
27 Polymer discs seeded with a monolayer of cells, and
28 branching fiber networks both satisfy these needs.

29 At the present time, a fibrillar structure is
30 preferred. The fibers may be round, scalloped,
31 flattened, star shaped, solitary or entwined with
32 other fibers. The use of branching fibers is based
33 upon the same principles which nature has used to
34 solve the problem of increasing surface area

1 compounds which enhance or allow ingrowth of the
2 lymphatic network or nerve fibers, and drugs, can be
3 incorporated into the matrix or provided in
4 conjunction with the matrix, as diagrammed in Figure
5 3.

6 The branching fibers 14 shown in Fig. 1, when 30
7 microns in diameter and 1.0 cm in length, can
8 theoretically support 125,000,000 cells. In the
9 example in which a liver organ is constructed, the
10 cell populations can include hepatocytes and bile duct
11 cells. Cells may be derived from the host, a related
12 donor or from established cell lines. Fetal cells
13 lines may be utilized since these cells are generally
14 more hardy than other cell lines.

15 In one variation of the method using a single
16 matrix for attachment of one or more cell lines, the
17 scaffolding is constructed such that initial cell
18 attachment and growth occur separately within the
19 matrix for each population. Alternatively, a unitary
20 scaffolding may be formed of different materials to
21 optimize attachment of various types of cells at
22 specific locations. Attachment is a function of both
23 the type of cell and matrix composition.

24 Although the presently preferred embodiment is
25 to utilize a single cell-matrix structure implanted
26 into a host, there are situations where it may be
27 desirable to use more than one cell-matrix structure,
28 each implanted at the most optimum time for growth of
29 the attached cells to form a functioning three-
30 dimensional organ structure from the different cell-
31 matrix structures. In some situations, it may be
32 desirable to prepare the implantation site by
33 initially exposing the cells at the site to a
34 biodegradable polymer matrix including compounds or

1 the systemic circulation through the hemiazygous
2 system. If this occurs, there would be portosystemic
3 channels through the implanted hepatic cells which may
4 allow for decompression of portal hypertension, a
5 complication leading to gastrointestinal bleeding in
6 patients with end-stage liver disease.

7 In the case of metabolic liver disease, where
8 the native liver is structurally normal and can drain
9 bile, appropriate hepatocytes on scaffolds can be
10 placed directly into the recipient liver. This
11 intrahepatic engraftment would occur in relation to
12 the normal host biliary system. The native liver
13 would then be a chimera of patient cells and donor
14 cells draining into the patient's biliary tree.

15 For this procedure to be successful, the
16 function of the implanted cells, both in vitro as well
17 as in vivo, must be determined. In vivo liver
18 function studies can be performed by placing a cannula
19 into the recipient's common bile duct. Bile can then
20 be collected in increments. Bile pigments can be
21 analyzed by high pressure liquid chromatography
22 looking for underivatized tetrapyrroles or by thin
23 layer chromatography after being converted to
24 azodipyrroles by reaction with diazotized
25 azodipyrroles ethylanthranilate either with or without
26 treatment with β -glucuronidase. Diconjugated and
27 monoconjugated bilirubin can also be determined by
28 thin layer chromatography after alkalinemethanolysis
29 of conjugated bile pigments. In general, as greater
30 numbers of functioning transplanted hepatocytes
31 implant, the levels of conjugated bilirubin will
32 increase. The same technique measuring monoconjugated
33 and diconjugated bilirubin can be performed in vitro
34 by testing the media for levels of these bilirubin

1 with hepatocytes; 23 with intestinal cells and
2 clusters; and 26 with pancreatic islet preparations.
3 The cells remained viable in culture, and in the case
4 of fetal intestine and fetal hepatocytes, appeared to
5 proliferate while on the polymer. After 4 days in
6 culture, the cell-polymer scaffolds were implanted
7 into host animals, either in the omentum, the
8 interscapular fat pad, or the mesentery. In 3 cases
9 of fetal intestinal implantation coupled with partial
10 hepatectomy, successful engraftment occurred in the
11 omentum, one forming a visible 6.0 mm cyst. Three
12 cases of hepatocyte implantation, one using adult
13 cells and two using fetal cells, have also engrafted,
14 showing viability of hepatocytes, mitotic figures, and
15 vascularization of the cell mass.

16 Materials and Methods

17 Polymers:

18 Three synthetic absorbable polymers were used to
19 fabricate filaments and discs as matrices for cell
20 attachment, growth, and implantation (Fig. 2).

21 1. Polyglactin. This polymer, developed as
22 absorbable synthetic suture material, a 90:10
23 copolymer of glycolide and lactide, is manufactured as
24 Vicryl® braided absorbable suture (Ethicon Co.,
25 Somerville, New Jersey) (Craig P.H., Williams J.A.,
26 Davis K.W., et al.: A Biological Comparison of
27 Polyglactin 910 and Polyglycolic Acid Synthetic
28 Absorbable Sutures. Surg. 141: 1010, (1975)).

29 2. Polyorthoesters. The specific polymer used
30 was: 3,9-bis(ethylidene-2, 4, 8, 10-tetraoxaspiro[5.5]
31 undecane copolymer with tran-1,4-cyclohexanedimethanol
32 and 1,6-hexandiol in a molar ratio 2:1:1, respectively
33 (SRI, California) (Heller J., Penhale W.H., Helwing

1 branching pattern relief, 10 mm in diameter and 0.5 mm
2 thick.

3 C. Filament Drawing. Filaments were drawn from
4 the molten polymer (30 microns in diameter). Small
5 flattened 1.0 cm. tufts were used for the experiments.

6 D. Polyglactin 910. Multiple fibers of 90:10
7 copolymer of glycolide and lactide converging to a
8 common base were fashioned from suture material of Q-
9 Vicryl[®] by fraying the braided end of the polymer.
10 These branching fiber clusters were approximately 1.0
11 cm. in height. The individual fibrils were 30 microns
12 in diameter.

13 Animals:

14 Young adult and fetal Sprague-Dawley rats and
15 C57 Bl/6 mice (Charles River Labs, Wilmington,
16 Massachusetts) were used as cell donors for all
17 experiments. The animals were housed individually,
18 allowed access to food and water ad lib, and
19 maintained at 12 hour light and dark intervals.
20 Animals were anesthetised with an IP injection of
21 pentobarbital (Abbott Labs, North Chicago, Illinois)
22 at a dose of 0.05 mg/g and supplemented with
23 methoxyflurane (Pitman-Moore, Inc., Washington
24 Crossing, New Jersey) by cone administration. Fetal
25 animals were harvested at 13, 17 and 20 days gestation
26 for use as liver, pancreas, and intestinal donors.
27 Young adult animals were used as liver and pancreas
28 donors and as recipients of the cell-scaffold
29 matrices.

30 Cell Harvest and Cell Culture

31 Liver:

32 After the induction of anesthesia, the abdomen
33 of young adult animals was shaved, prepped with
34 betadine, and opened using sterile technique. The

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1 animals of the appropriate gestation. The intact
2 uterus with multiple fetuses was transferred in saline
3 to a sterile room, equipped with a dissection
4 microscope. Individual fetuses were opened and the
5 liver, intestine, and pancreas were harvested and
6 pooled. Organs were then transferred to a sterile
7 hood for cell isolation. The tissues were minced,
8 treated with a 0.025% Type II collagenase, and
9 dispersed into cell suspensions.

10 Pancreas:

11 After the induction of anesthesia, the abdomen
12 of young adult animals was shaved, prepped with
13 betadine, and opened in the midline using sterile
14 technique. The common bile duct was isolated, and the
15 pancreas visualized. 2.5 cc.'s of 2.0% Type II
16 collagenase (BCA/Cappel Products, West Chester,
17 Pennsylvania), was infused into the pancreas by
18 injection into the common bile duct using the
19 technique described by Gotoh et al. (Gotoh M., Maki
20 T., Kiyozumi T., et al.: An Improved Method of
21 Isolation of Mouse Pancreatic Islets. Trans. 40; 4,
22 pp. 436-438, 1985). After 5 minutes, the pancreas was
23 transferred to a sterile hood for islet cell
24 isolation. Briefly, the tissue was placed into a 25%
25 Ficoll solution and layered under a discontinuous
26 Ficoll gradient (23, 21, 11%) and centrifuged at 800 x
27 g. for 10 minutes. Islets which aggregated at the 21-
28 11% interface were washed with cold Hank's solution
29 and centrifuged at 320 x g. 3 times. The islets were
30 resuspended in RPMI 1640 (Gibco, Grand Island, New
31 York) media-supplemented with 10% fetal calf serum,
32 and overlaid onto polymer scaffolds. Fetal animals
33 were harvested as donors as described above.

1 fluoresceine isothiocyanate (FITC) antisera labelled to
2 the appropriate monoclonal antibody, for example, the
3 HY antigen or other markers of hepatocyte membranes,
4 are applied individually to separate moist biopsy
5 sections. They are incubated at room temperature for
6 30 minutes in a moist chamber. Following rinses with
7 PBS the sections are cover-slipped with a glycerol-PBS
8 mixture and examined using an immunofluorescence
9 microscope (Leitz) with epi-illumination and a high
10 pressure mercury lamp as the light source.

11 Electron microscopy: samples for electron
12 microscopy are obtained from fresh tissue and fixed in
13 2% glutaraldehyde, post-fixed in 1% osmiumtetroxide,
14 dehydrated in graded alcohols, and imbedded in epon-
15 8:12. One micron thick section of the plastic
16 imbedded tissue are made from areas of interest.
17 Selected blocks are trimmed, ultrathin sections made,
18 and stained with uranyl acetate and lead citrate, and
19 examined with a Phillips 300X electron microscope.
20 Scanning electron microscopy (SEM): After hepatocytes
21 are isolated and attached to the appropriate polymer,
22 they are incubated for the appropriate interval.
23 After culture, samples are prepared for SEM by
24 incubating in a 50:50 solution of 2% glutaraldehyde
25 phosphate buffer solution for 1 hour, the samples are
26 then rinsed 4 times in PBS for 10 minutes per rinse to
27 remove excess glutaraldehyde solution. Samples are
28 dehydrated using progressively increasing ethanol
29 solutions. Samples are then placed in a critical
30 point dryer where ethanol is exchanged for liquid CO₂.
31 Temperature is gradually increased to the critical
32 point, ensuring dehydration. The samples are then
33 coated with a thin layer of gold and placed under high
34 vacuum in the scanning electron microscope.

1 phase and a chronic foreign body reaction to the
2 implanted polymers. The intensity of inflammation
3 varied with the polymer type tested: polyanhydride
4 elicited the most severe acute and chronic response
5 although the inflammation surrounding branching fibers
6 of either polyorthoester or polyglactin appeared
7 proportionately greater than the disc configuration
8 because of the greater surface area of exposed foreign
9 material to host.

10 Histologic examination of liver cell implants in
11 3 animals showed evidence of successful engraftment of
12 hepatocytes at seven days, as shown by Figure 7.
13 Small clusters of healthy appearing hepatocytes were
14 seen with bile canaliculi between adjacent cell
15 membranes and some areas demonstrated mitotic figures.
16 The cells were surrounded by an inflammatory response
17 and blood vessels coursed around and through the cell
18 clusters. Polymer material was seen immediately
19 adjacent to the cells.

20 Successful engraftment of intestinal cells and
21 clusters were observed in 3 animals. Histologic
22 findings were similar to the hepatocyte implants. On
23 gross examination of the implant at 7 days, a cystic
24 structure approximately 6.0 mm in length was found at
25 the implant site with polymer fibers displayed within
26 its wall (Figure 10). Microscopic examination
27 revealed well differentiated intestinal epithelium
28 lining the cavity with mucous and cellular debris
29 within the lumen, shown in Figure 11. One wall of the
30 cyst contained polymer fibers, blood vessels, and
31 inflammatory cells immediately adjacent to the
32 intestinal epithelium. The other wall included a
33 muscular coating which suggested that the polymer held
34 a small minced piece of fetal intestine as the origin

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1 fibers seeded with bovine aortic endothelial cells in
2 a biomatrix. The cells can be seen migrating off the
3 polymer into the matrix in a branch-like orientation.
4 Figure 14 is a photograph of bovine aortic endothelial
5 cells attached to polymer fibers after one month in
6 culture. These cells have been shown to reform
7 structure. Their ability to do so depends upon the
8 environment in which they are placed and the degree of
9 alteration they have undergone. In addition to the
10 the bile duct cells which formed tubules in vitro as
11 shown in Figure 6, the aortic endothelial cells
12 attached to polymer fibers formed branching tubule
13 structures after one month in culture. As the polymer
14 fibers resorbed, the cells maintained their
15 orientation, indicating that they secreted their own
16 matrix to maintain their geometric configuration.

17 Figure 15 is a phase contrast photomicrograph
18 showing polymer fibers coated with mouse fetal
19 fibroblasts. The fibroblasts can be seen streaming
20 off the polymer fibers in a straight line onto the
21 culture dish. This indicates that cell-cell
22 orientation cues have been maintained as they migrate
23 off the polymer fiber.

24 Figure 16 is a phase contrast photomicrograph of
25 polymer fibers coated with mouse fetal fibroblasts.
26 These fetal fibroblasts have migrated off of the
27 polymer through media and have attached at the bottom
28 of the tissue culture plate. This shows that a living
29 tissue bridge has been created between the polymer
30 fiber and the tissue culture bottom by fetal
31 fibroblasts, indicating their spatial organization.

32 These studies demonstrate that cells of liver,
33 intestine, and pancreas will successfully attach and
34 remain viable on polymers in cell culture and that

1 hour. A combined coating using gelatin and gum arabic was tested. Collagen coated polymers were prepared by covering the polymer with a Type IV collagen and lyophilizing this polymer-collagen material overnight. Some collagen coated samples were immersed in phosphate buffer for one hour. All samples were examined by SEM to determine uniformity of coating. All samples were sterilized using UV exposure under the sterile hood for 8-12 hours. Cells were then added for cell attachment studies.

11 Cell Attachment Studies.

12 Cell polymer samples were examined by phase contrast microscopy and SEM using the following sample preparation technique. Samples were fixed by immersion in 50:50 2% gluteraldehyde:phosphate buffer for 1 hour and then rinsed x 3 for 20 minutes with phosphate buffer. They were then dehydrated in progressively increasing concentrations of ethanol solutions (70%, 80%, 90%, 95%) for 20 minutes each, immersed in absolute alcohol overnight, dried by critical point drying with liquid CO₂ and coated with gold.

23 Isolation and purification of Pancreatic Islet Cells.

24 Young adult mice were anesthetized and underwent a midline abdominal incision using sterile technique. The common bile duct was isolated and cannulated with a 30 gauge needle. 2.5 cc. of Type IV collagenase was slowly infused through the common bile duct with a clamp on the duodenum so that there would be retrograde flow into the pancreatic duct. The pancreas was then removed and digested with collagenase for 45 minutes at 37°C. The pancreas was then washed with cold Hank's solution and pancreatic tissue passed through a nylon mesh filter. The islets

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1 attachment of pancreatic cells (islets and
2 fibroblasts) on vicrylTM after two weeks in culture.
3 Maximum attachment occurs with polymer coated with
4 crosslinked or uncrosslinked 11% or 1.5% gelatin and
5 collagen. Very little attachment of these cells to
6 polyorthoester and polyanhydride samples was observed.
7 Table V is the attachment of islet cells after two
8 weeks in culture, with maximum attachment again
9 occurring with polymer coated with collagen.

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TABLE II

3T3 CELLS ON POLYORTHOESTER

<u>Polymer</u>	<u>Attachment after 2 days</u> Some degradation	<u>Attachment after 5 days</u> Considerable degradation
Control (untreated, with no cells)	1	1
Untreated	1	1
Agar (5%)	1	1
Agarose (6.7%)	2	4+
Gelatin (11%) crosslinked	4	2
Gelatin (11%) gum 11% crosslinked	1	1
Gum arabic (11%)	1	0
pH 4, 2 days	2	1
pH 4, 5 days	1	1
pH 4, 7 days	3	2
pH 7, 2 days	4	3
pH 7, 5 days	2	1
pH 7, 7 days	4+	4+
pH 10, 2 days	0	0
pH 10, 5 days	4	3
pH 10, 7 days		

Scale

- 0 No viable cells
- 1 Minimal cell attachment
- 2 Moderate cell attachment
- 3 Good cell attachment
- 4 Better cell attachment
- 5 Excellent cell attachment

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TABLE IV
 PANCREATIC CELLS ON VICRYL[®] AFTER 2 WEEKS
 IN CULTURE (MIXTURE OF ISLETS AND FIBROBLASTS)

<u>Polymer</u>	<u>Attachment</u> Little, if any degradation
Control (untreated, no cells)	0
Untreated	1
Agar (2%)	1
Agarose (2%)	4
Gelatin (11%) crosslinked	4+
Gelatin (11%)	2
Gelatin (1.5%) crosslinked	4+
Gelatin (1.5%)	1
Gum arabic (1.5%)	1
Gelatin (1.5%)/Gum arabic (1.5%) crosslinked	2
Gelatin (1.5%)/Gum arabic (1.5%)	4++
Collagen	3
Collagen - phosphate buffer	0
pH 4, 2 days	0
pH 4, 4 days	1
pH 7, 2 days	2
pH 7, 4 days	1
pH 10, 2 days	

Scale

- 0 No viable cells
- 1 Minimal cell attachment
- 2 Moderate cell attachment
- 3 Good cell attachment
- 4 Better cell attachment
- 5 Excellent cell attachment

1 The method of the present invention is highly
2 versatile and useful both in vivo and in vitro. For
3 example, cells on polymer fibers embedded in Matrigel
4 can be used to create three-dimensional organ
5 structures in vitro. For in vivo applications, the
6 polymer structure is tailored to fit the cells so that
7 the desired function and structure is obtained after
8 implantation, and so that cell growth, proliferation
9 and function can be achieved initially in cell
10 culture. The criteria for successful growth and
11 implantation is when the transplant demonstrates
12 functional equivalency to the organ which it is
13 replacing or supplementing. For example, a functional
14 kidney would not necessarily have to manufacture renin
15 as long as it functions as an effective dialysis
16 apparatus, removing concentrated low molecular weight
17 materials from the bloodstream. A functional liver
18 may only need to produce protein such as coagulation
19 factors and excrete bile. For this purpose the liver
20 transplant could be implanted in the omentum, the
21 fatty, highly vasculated membrane adjacent to the
22 small intestine. A functional intestine should be
23 able to absorb sufficient nutrients to sustain life.
24 This could be in the form of caloric solutions rather
25 than normal "foodstuffs".

26 "Secretory" organs in addition to a liver or a
27 pancreas can be made by applying the same method of
28 selecting secretory cells, constructing a matrix,
29 culturing the cell on the matrix, and implanting the
30 cell-matrix structure into an area which promotes
31 vasculature of the cell-matrix structure.

32 As demonstrated in Figure 19, "organs" other
33 than secretory organs can be made using the method of
34 the present invention. Nerves may be constructed

1 vascularization. Unless the cells are more or less
2 equally exposed to the media, with as shallow of a
3 concentration gradient as possible, this will not
4 occur. As the cells multiply, the passage of
5 nutrients, wastes, and gases to and from the cells
6 becomes limited and the cells farthest from the media
7 die. Since the artificial skin implants were
8 immediately placed on the underlying tissue so that
9 capillary growth into the matrix begins prior to any
10 significant increase in cell density, this has not
11 previously been a consideration.

12 The concept of Chimeric Neomorphogenesis hinges
13 upon the ability of cells to be nourished by diffusion
14 until vascular ingrowth of the growing cell mass
15 occurs. It was hypothesized that solid implants of a
16 cell-matrix configuration using collagen or gelatin
17 seeded with cells are limited in size by the physical
18 constraints of diffusion. Others are presently using
19 complex natural matrices seeded with cells to produce
20 "organ equivalents". One is a collagen gel that
21 appears to be a hydrated solution of Type I collagen.
22 The following experiment tests the ability of this
23 hydrated collagen to allow diffusion of nutrients to a
24 cell population.

25 Bovine capillary endothelial cells were plated
26 in gelatin coated 24 well tissue culture dishes and
27 allowed to attach overnight. The initial cell number
28 was 1×10^5 cells. The following day the cells were
29 overlaid with different volumes of collagen Type I at
30 a final solution of 0.32%. A standard volume of media
31 was placed over the collagen so that the distance of
32 nutrient source varied to the cells. The media was
33 optimized for growth of bovine capillary endothelium.
34 Dulbecco's minimal essential media, 10% calf serum,

1 constraints. One would expect that an implant of less
2 than 1 cm³ would result in cell viability at the
3 periphery of the implant to a depth of 3-5 mm.
4 However, the cells in the center of the implant would
5 not remain viable because of limitation of nutrition,
6 diffusion, as well as gas exchange. One can envision
7 large flat gels with very small thicknesses of 5-10 mm
8 would allow larger implants to occur. However, this
9 two dimensional solution may have geometric
10 constraints for implantation. It is also clear that
11 by increasing cell density, diffusion would be more
12 limited, and, therefore, the distances would be
13 commensurately smaller.

14 Although this invention has been described with
15 reference to specific embodiments, variations and
16 modifications of the method and means for constructing
17 artificial organs by culturing cells on matrices
18 having maximized surface area and exposure to the
19 surrounding nutrient-containing environment will be
20 apparent to those skilled in the art. Such
21 modifications and variations are intended to come
22 within the scope of the appended claims.
23

1 host at the location where said cell-matrix structure
2 is to be implanted.

3 6. The method of claim 4 further comprising
4 implanting additional cell-matrix structures having
5 different cell populations in conjunction with the
6 first cell-matrix structure.

7 7. The method of claim 3 further comprising
8 removing lymphocytes from the cell population prior to
9 seeding.

10 8. The method of claim 3 further comprising
11 modifying the cells to alter the antigen expression on
12 the cell surface.

13 9. The method of claim 4 further comprising
14 selecting cells of a tissue type compatible with the
15 host's cells.

16 10. The method of claim 1 further comprising
17 providing compounds selected from the group consisting
18 of nutrients, cofactors, growth factors, compounds
19 stimulating angiogenesis, immunomodulators, inhibitors
20 of inflammation, regression factors, factors
21 stimulating differentiation and dedifferentiation,
22 biologically active molecules stimulating lymphatic
23 network ingrowth, factors enhancing nerve growth and
24 drugs.

25 11. The method of claim 1 further comprising
26 selecting the biocompatible material for the group
27 consisting of polyorthoesters, polyanhydrides,
28 polyglycolic acid, basement membrane components, agar,
29 agarose, gelatin, gum arabic, collagen types I, II,
30 III, IV, and V, fibronectin, laminin,
31 glycosaminoglycans, and complex mixtures thereof.

32 12. The method of claim 1 wherein the
33 biocompatible material is selected from the group of

1 18. The matrix of claim 16 further comprising
2 an overlayer enhancing cell attachment.

3 19. The matrix of claim 16 wherein said second
4 material is selected from the group of materials which
5 enhance adhesion of cells to the surface of said
6 matrix consisting of agar, agarose, gelatin, gum
7 arabic, basement membrane material, collagens types I,
8 II, III, IV, and V, fibronectin, laminin,
9 glycosaminoglycans, and complex mixtures thereof.

10 20. The matrix of claim 16 wherein the matrix
11 is disc shaped and has specifically contoured
12 depressions for cell attachment.

13 21. The matrix of claim 16 wherein said matrix
14 is a fibrous structure.

15 22. The matrix of claim 21 wherein said fibrous
16 structure includes hollow fibers.

17 23. The matrix of claim 21 wherein said fibrous
18 structure includes solid fibers.

19 24. The matrix of claim 16 wherein said matrix
20 is biodegradable.

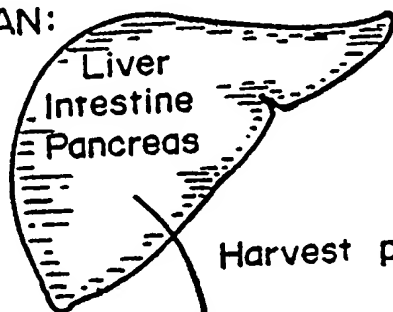
21 25. The matrix of claim 16 wherein said matrix
22 is configured as spicules.

23 26. The matrix of claim 16 further comprising
24 compounds selected from the group consisting of
25 nutrients, growth factors, cofactors, compounds
26 stimulating angiogenesis, immunomodulators, inhibitors
27 of inflammation, regression factors, factors
28 stimulating differentiation and de-differentiation,
29 biologically active molecules stimulating lymphatic
30 network ingrowth, factors enhancing nerve growth,
31 drugs and combinations thereof.

32 27. The matrix of claim 16 wherein said matrix
33 is configured to provide separate areas of attachment
34 for cells of different origin.

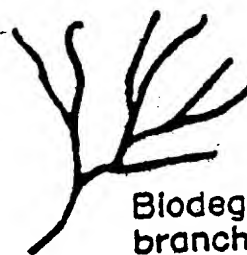
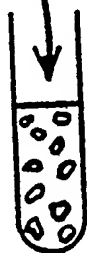
1 / 14

ORGAN:

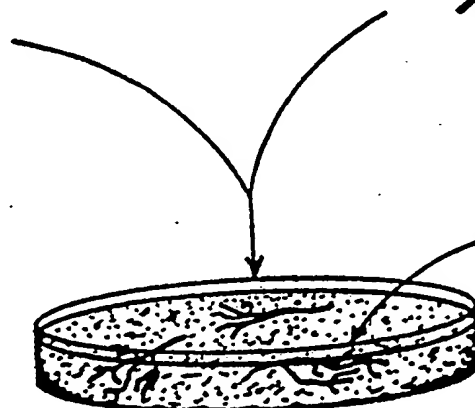


Harvest parenchymal cells

Cell suspension



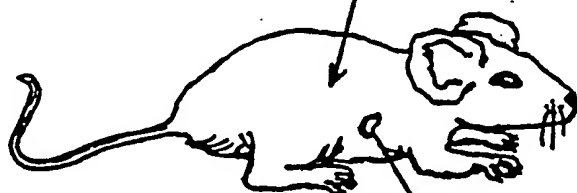
Biodegradable branching polymer



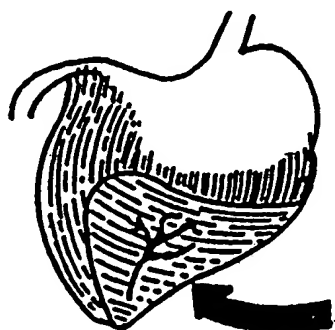
Polymer Cell scaffold

Culture 4 days

Implant into animal



Partial hepatectomy



Under folded omentum

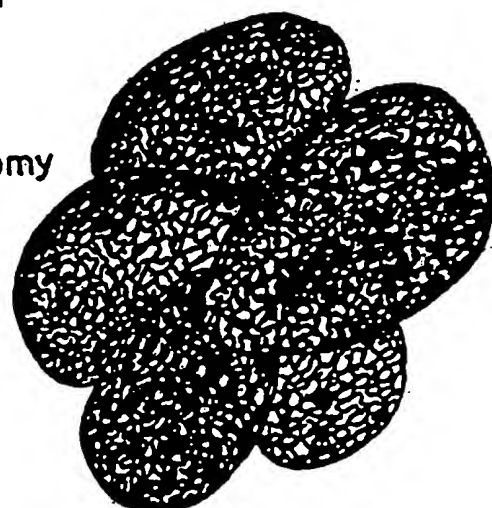


FIG. 1

3 / 14

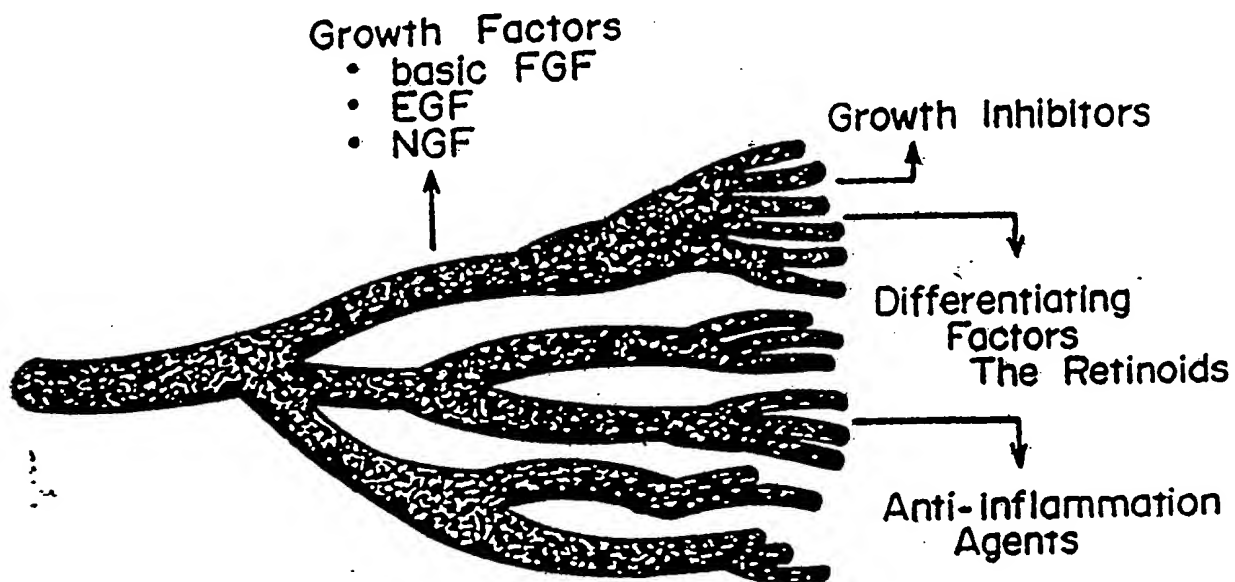


FIG.3

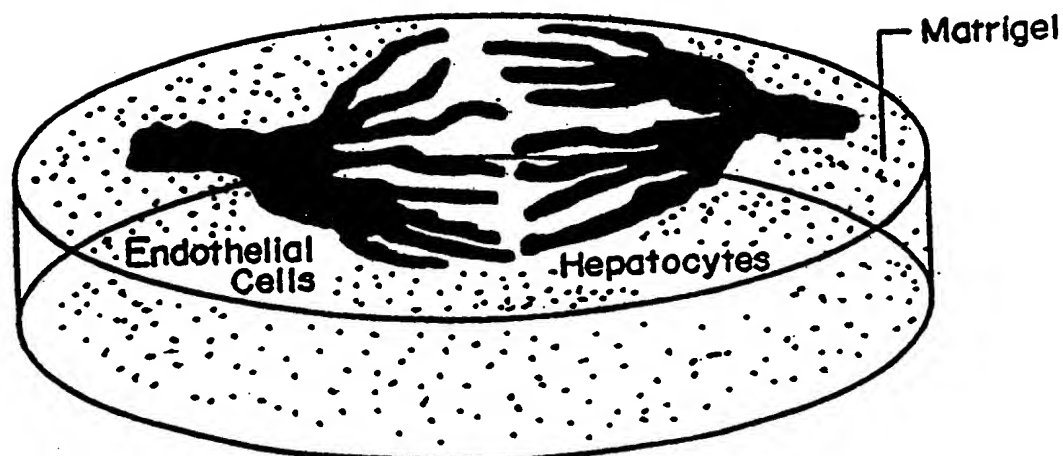


FIG.4

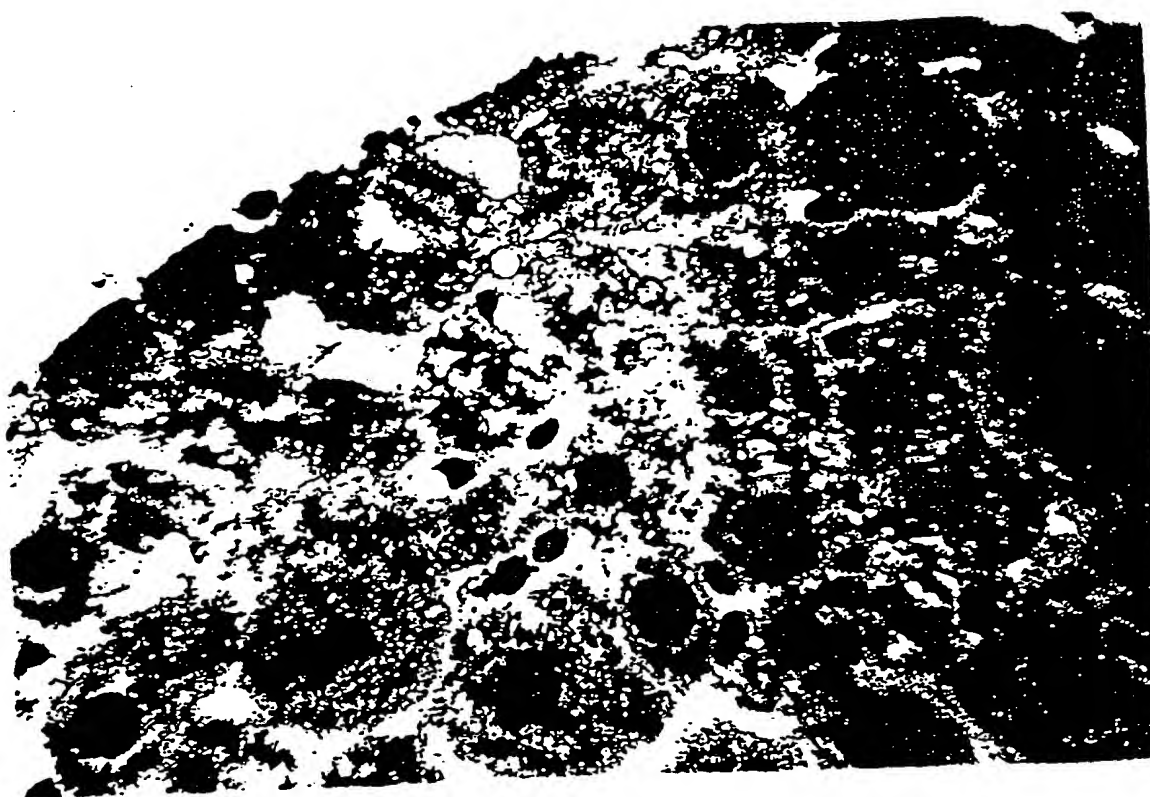


FIG. 7

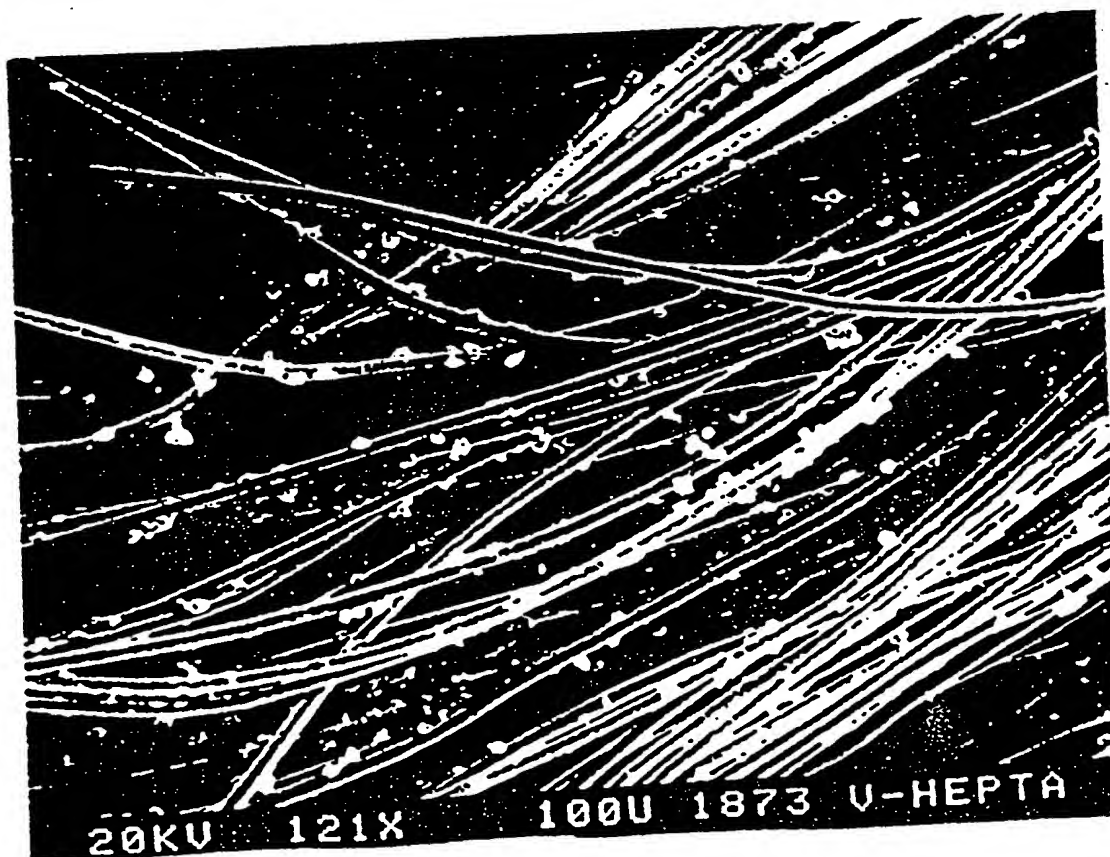


FIG. 8



FIG. 11



FIG. 12



FIG.15

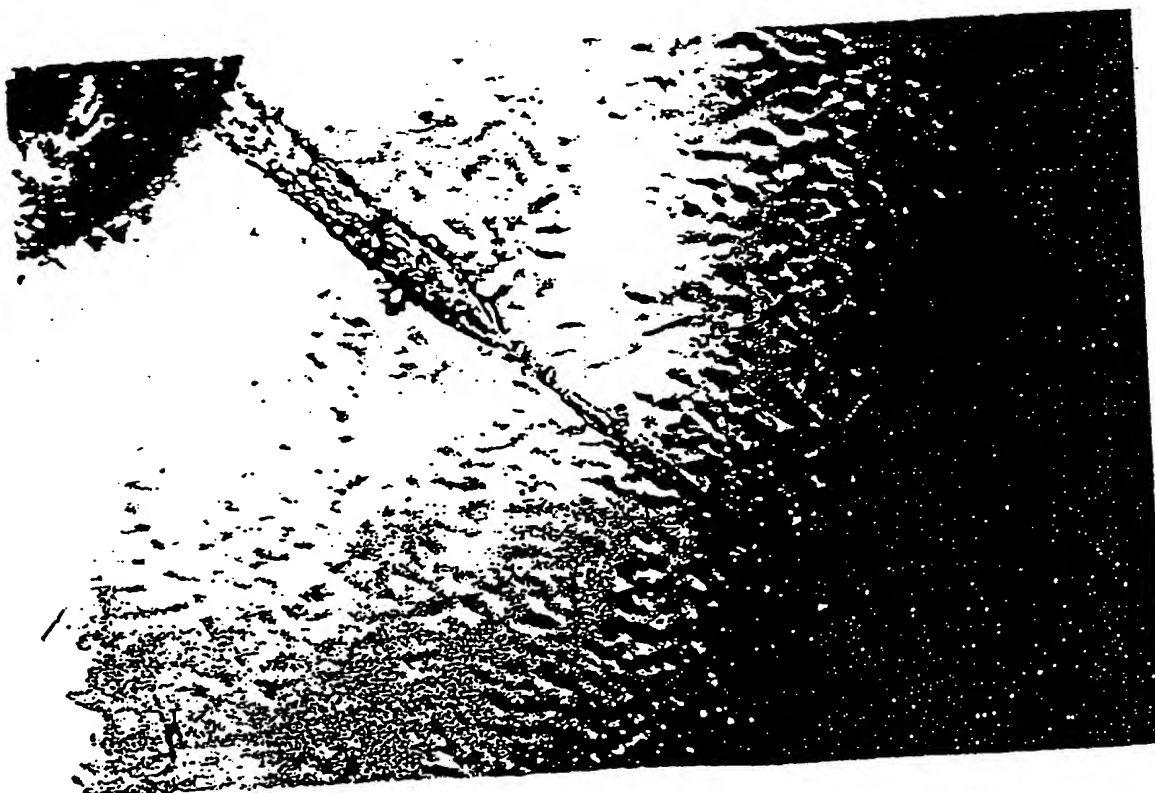


FIG.16

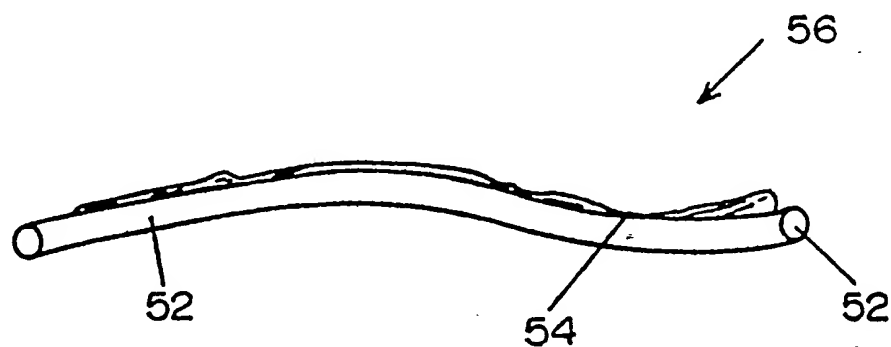


FIG. 19

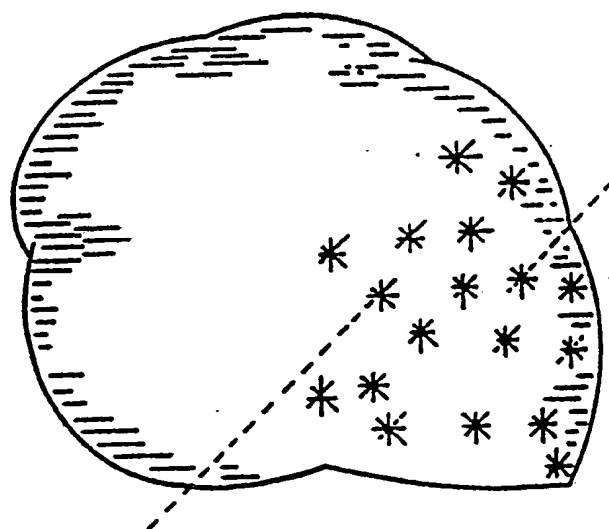


FIG. 20a

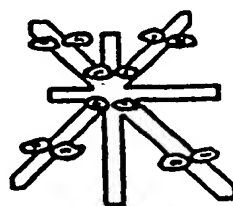


FIG. 20b

13/14

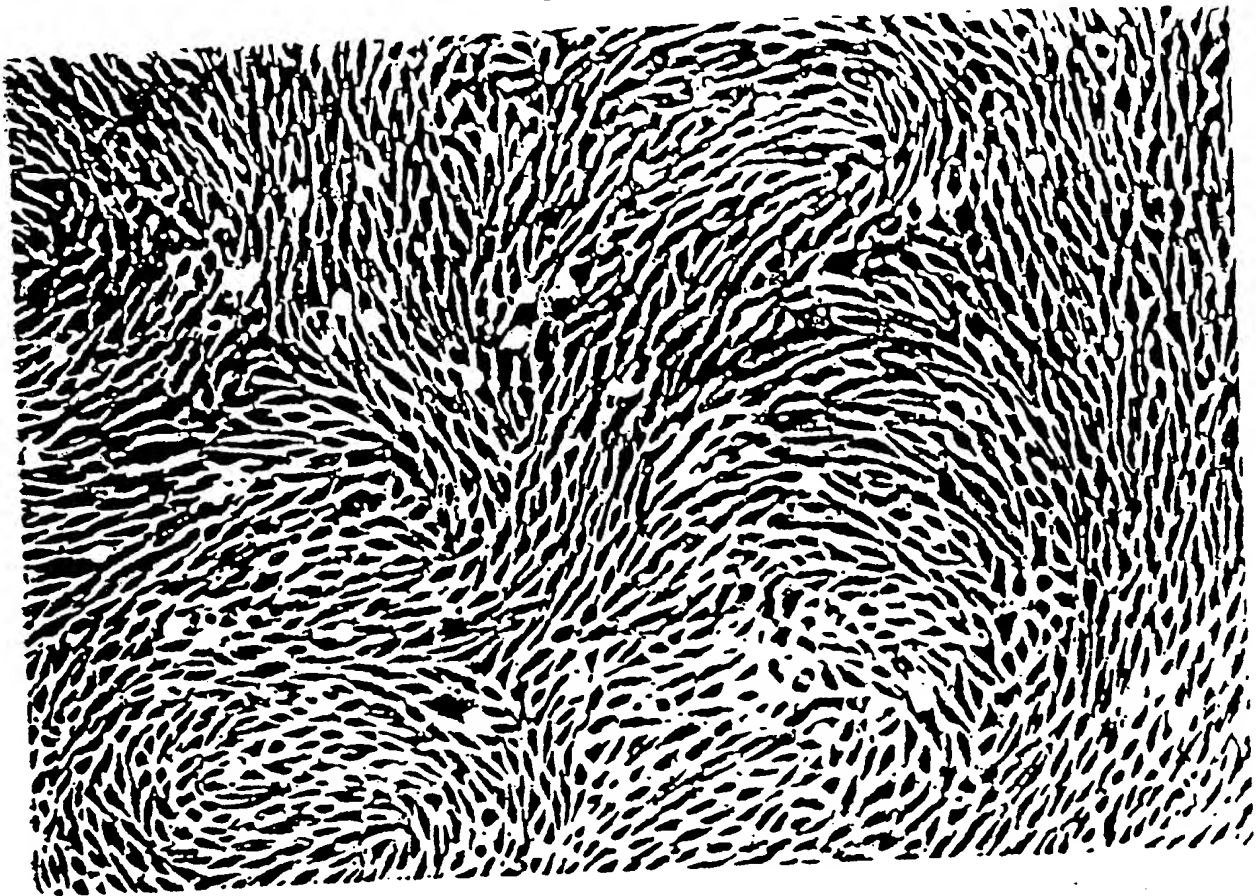
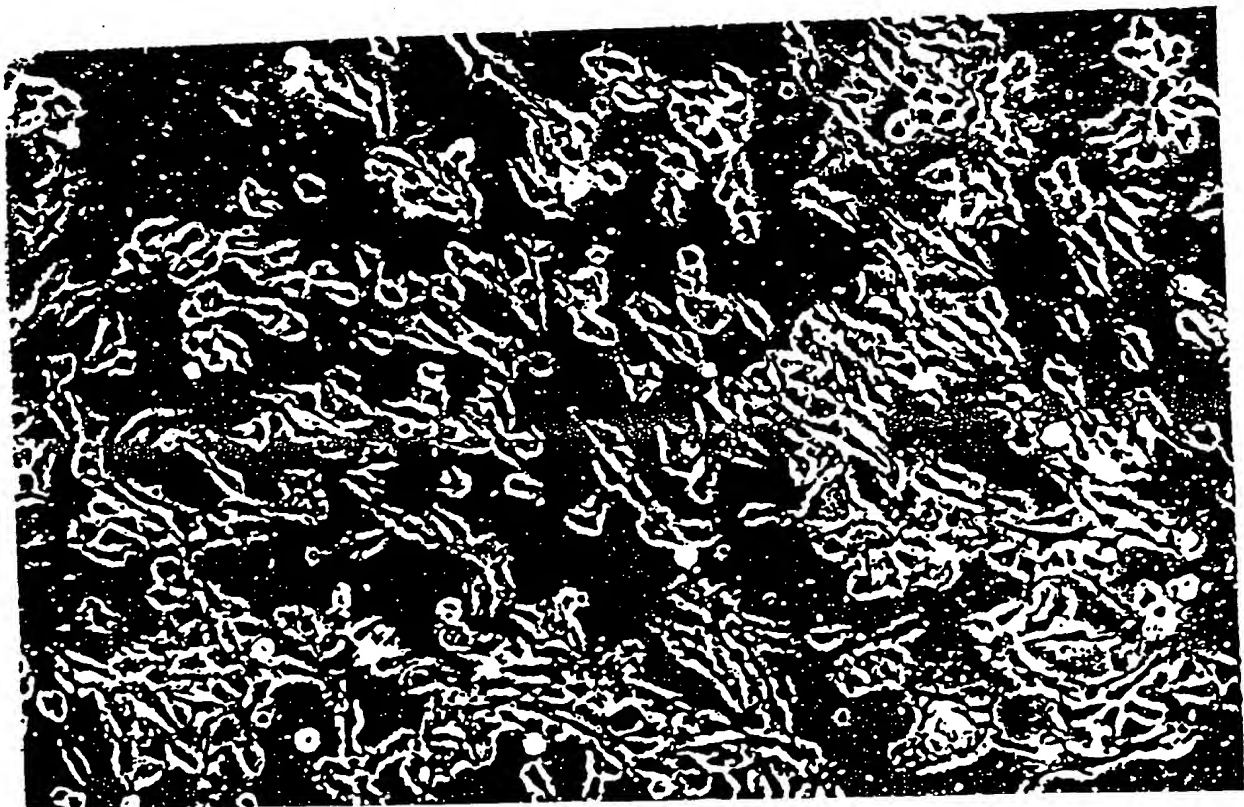


FIG.22a



INTERNATIONAL SEARCH REPORT

International Application No. PCT/US87/03091

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) According to International Patent Classification (IPC) or to both National Classification and IPC	
IPC ⁴ A61B 19/00; CL2N 5/00	U.S. Cl. 435/240.21
II. FIELDS SEARCHED	
Minimum Documentation Searched *	
Classification System	Classification Symbols
U.S. Cl. 128/1R; 435/240.21, 240.23, 240.243; 424/422, 423, 486	
Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched *	

III. DOCUMENTS CONSIDERED TO BE RELEVANT ¹⁴		
Category *	Citation of Document, ¹⁵ with indication, where appropriate, of the relevant passages ¹⁷	Relevant to Claim No. ¹⁸
X Y	US 4,427,808 STOL, 24 JAN 1984	<u>1-4,9-12,14-17,19,21,24,26</u> 1-29
X Y	US 4,485,097 BELL, 27 NOV 1984	<u>1-4,9-13,15-17,19,21,24,26</u> 1-29
X Y	US 4,458,678 YANNAS, 10 JUL 1984	<u>1-4,8-12,15-19,24,26</u> 1-29
X Y	US 4,060,081 YANNAS, 29 NOV 1977	<u>1,2,11,12,16-19,24</u> 1-29
X Y	US 4,553,272 MEARS, 19 NOV 1985	<u>1,10,16,18,26</u> 1-29
X Y	US 4,559,304 KASAI, 17 DEC 1985	<u>1-3,11,12,16,17,19,24</u> 1-29 1-29 1-29 1-29
Y	US 4,528,265 BECKER, 9 JUL 1985	1-29
Y	US 4,444,887 HOFFMANN, 24 APR 1984	1-29
P, Y	US 4,645,669 REID, 24 FEB 1987	1-29

- * Special categories of cited documents: ¹³
- "A" document defining the general state of the art which is not considered to be of particular relevance
 - "E" earlier document but published on or after the international filing date
 - "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
 - "O" document referring to an oral disclosure, use, exhibition or other means
 - "P" document published prior to the international filing date but later than the priority date claimed

- "T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
- "X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step
- "Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.
- "A" document member of the same patent family

IV. CERTIFICATION	
Date of the Actual Completion of the International Search ¹ 22 MAR 1988 International Searching Authority ¹ ISA/US	Date of Mailing of this International Search Report ¹ <div style="font-size: 1.5em; font-weight: bold; text-align: center;">05 APR 1988</div> Signature of Authorized Officer ¹⁶ <i>Catherine S. Kilby</i> Catherine S. Kilby